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A molecular approach to understanding root bud dormancy in leafy spurge

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Leafy spurge is a tenacious perennial weed of the Northern Plains. This plant maintains a perennial growth cycle by controlled production and growth of numerous underground adventitious buds. We are using molecular tools to identify signaling pathways that control underground adventitious bud growth and development in leafy spurge. Toward this end, we have used three techniques to identify genes that are differentially expressed concomitantly with the breaking of quiescence in underground buds of leafy spurge. These techniques include differential display of cDNAs, random cloning and sequencing of genes expressed in growing buds, and microarray technology. To date, we have identified more than 16 genes that are differentially expressed in underground buds of leafy spurge during dormancy break and growth initiation. A detailed expression analysis of these genes will allow them to be grouped by their responses to various signals known to play a role in control of underground bud growth. This information will be used to identify key *cis*-acting elements involved in the regulation of these genes. How such information on signal transduction processes may be used for developing new weed control strategies by the identification of novel target pathways and development of DNA-based herbicides is presented.

Nomenclature: Leafy spurge, *Euphorbia esula* L. EPHES.

Key words: Reproduction, vegetative propagules.

Leafy spurge is a deep-rooted perennial weed that primarily infests range and recreational lands in the Northern Great Plains of the United States and Canada. Like many perennial weeds, leafy spurge propagates through the production and growth of numerous underground adventitious buds. Once formed, these buds enter a quiescent state and remain dormant until growth is reinitiated by separation from or death of the aerial portion of the plant.

Little is known about the molecular mechanisms that control dormancy and growth of underground buds and seeds of leafy spurge or other perennial plants. However, physiological studies have shown that correlative inhibition of leafy spurge underground buds is maintained by at least two separate signals. One of the signals is produced in young expanding leaves and meristems of the plant and is likely auxin (Horvath 1998). The other signal is produced in the mature leaves, requires photosynthesis for its production and transport, and can be overcome by exogenous application of gibberellic acid (GA) (Horvath 1999). This second signal is likely a sugar (Chao et al. 2000).

It is not known how these physiological signals control the growth of underground buds. There is some evidence that auxin acts indirectly through products of the *Rms1* genes to inhibit growth of buds below the apical meristem in pea (*Pisum sativum* L.) (Beveridge et al. 2000). However, how this interaction functions to prevent growth of axillary buds is not known. Interestingly, auxin appears to play a positive role during bud growth in some systems. It has been shown that auxin production within the quiescent axillary buds of pea increases concomitantly with resumed growth (Stafstrom et al. 1998). Auxin also plays an important role in regulating the level of key cell cycle components in Arabidopsis (*Arabidopsis thaliana* L.) (Leyser et al. 1993).

The role of sugar in the control of growth and development is just beginning to be understood. Among their other roles, both sugar and cytokinins have been shown to induce G1 cyclin expression in tissue culture (Gaudin et al. 2000; Soni et al. 1995). Also, sugar has been shown to inhibit GA signaling in several different plant systems (Chao et al. 2000; Perata et al. 1997; Xu et al. 1998). GA also is suspected of playing a key role in the control of cell division and elongation as well as in paradormancy phenomenon (also known as apical dominance or correlative inhibition) (Cline 1991; Gendreau et al. 1999; Sauter et al. 1995). Thus, it seems likely that sugar may, in part, be affecting cell cycle control through its interactions with GA. Additional supporting evidence for an interaction of sugar with GA signaling comes from recent findings that several sugar insensitive mutants are allelic with several genes known to play a role in abscisic acid (ABA) signal transduction (Huijser et al. 2000; Laby et al. 2000). Antagonistic crosstalk between ABA and GA signaling is a well-known phenomenon.

To better understand how these signals control growth in leafy spurge, we have undertaken the cloning of genes that are differentially expressed concomitantly with the initiation of underground bud growth. These differentially expressed genes are likely to contain specific sequences (*cis*-acting elements) within their regulatory regions (promoters, etc.) that are responsive to the signals controlling growth and dormancy. Identifying such sequences and the proteins that interact with them will be critical for deciphering the signaling pathways involved in controlling their expression and will likely provide clues as to the signaling pathways controlling growth and dormancy in underground buds of leafy spurge. Evidence for the effectiveness of this procedure comes from studies that compared the promoter sequences of cold-regulated glycine-rich RNA-binding protein genes from leafy spurge, Arabidopsis, and *canola* (*Brassica napus* L.) which identify a recognized cold response *cis*-acting element (Horvath and Olson 1998). In this paper, we demonstrate the differential expression of a number of genes likely to be controlled by GA and cell cycle responses. This information is used to develop a model for controlling initiation of underground bud growth.

Materials and Methods

Plant Material

Plants used for these experiments were propagated from shoot cuttings as a small group of plants which were originally isolated from a wild leafy spurge population in North Dakota. Shoot cuttings were placed in Sunshine mix[1] and grown in 2.5 by 20 cm cones in a greenhouse under an 18-h photoperiod at 28 ± 4 C for 3 mo. All plants used consisted of single stems with 70 to 100 leaves and an average of 56 (SD = 20) root buds per plant. All harvested tissues were immediately frozen in liquid N_2 and stored at − 80 C until RNA extractions were initiated. All experiments were repeated with at least two separate sets of 14 to 21 plants treated at different times.

Plant Treatments

To study the temporal expression pattern of the genes, the crown and aerial portion of the plants were excised and all underground buds greater then 0.25 mm in length were harvested at 12-h intervals. To reduce the possibility of observing circadian-regulated changes in gene expression, plants were excised so that harvest could be initiated between 0800 and 1000 h daily.

To study the effects of various plant organs on underground bud growth, specific plant organs were removed as previously described (Horvath 1999). Briefly, plants were either left intact or the entire aerial portion of the plant was excised to the base of the crown. Alternatively, the apical meristem and top 10 cm of the plants were removed (meristemless) or they were stripped of either mature leaves (leafless) or axillary buds (budless). All distinguishable underground buds 0.25 mm or larger were harvested 3 d following treatment.

To test the effects of hormones on gene expression in underground buds, plants were watered once with 25 ml of a 0.5% Tween 20 solution with or without 1 mM GA, and the crown was abraded and ringed with lanolin paste that was or was not supplemented with 1% wt/v *N*-1-naphthylphthalamic acid (NPA). All distinguishable underground buds 0.25 mm or larger were harvested 3 d following treatment.

Gene Identification Procedures

Microarray analysis was done using methods developed for the *Arabidopsis* Functional Genomics Consortium (Girke et al. 2000). *Arabidopsis* microarrays for each experiment were hybridized with labeled cDNA probes produced from independently isolated sets of leafy spurge RNA. Sequence analysis of all cDNA (identified as differentially expressed or randomly chosen from the cDNA library) was done by Iowa State University's DNA Sequencing Facility. Differential display of cDNA was done using the GenHunter RNAmap[1] differential display kit according to manufacturer's protocols.

Northern Blot Analysis

RNA was collected using the Pine Tree Extraction method (Chang et al. 1993). Total RNA (50 μg) was separated on denaturing agarose gels and blotted according to standard techniques (Sambrook et al. 1989). DNA probes were prepared by polymerase chain reaction amplification of designated cDNA, followed by the isolation of the resulting fragment after separation on agarose gels. Radiolabeled probes representing genes were prepared and hybridized to the various blots (5 × standard saline citrate (SSC)/50% formamide) at 42 C overnight. Blots were washed four times in 2 × SSC, 0.2% sodium dodecyl sulfate at room temperature for 5 min each and then two times at 65 C for 15 min each. The resulting hybridizations were visualized on a Packard Instant Imager®. Linearity was maintained for all of the images presented. Genetic material used for generating probes for these studies were obtained from a leafy spurge EST-database developed from a cDNA library made using underground buds harvested 3 d after defoliation. Each northern hybridization experiment was repeated at least twice with independently isolated sample sets.

Results and Discussion

Cloning of Differentially Expressed Genes

As described in Materials and Methods (Gene Identification Procedures), three methods were used to identify differentially expressed leafy spurge genes in underground buds as they are released from dormancy: differential display of cDNA; sequence analysis of genes from an EST database and comparison with genes with known functions from other plant systems; and comparison of expression analysis of leafy spurge leaf and meristem tissue using *Arabidopsis* microarrays. These methods resulted in the identification of at least 16 different genes induced during dormancy break in underground buds of leafy spurge (Table 1). These data indicate that all the three methods are capable of identifying growth-responsive genes from leafy spurge. Additionally, there were a number of genes identified by differential display or by sequence similarity to previously characterized genes that were likely to be differentially expressed and that were present on the *Arabidopsis* microarray. These genes included *Histone 2A* and *Histone H3,* a homologue of *At103* (a leucine-zipper–containing gene previously shown to be regulated in germinating seeds by phytochrome), *Alpha-tubulin, BiP* (a gene coding for a glucose-responsive chaperon-binding protein gene), and a homologue of *GASA4* (a GA-responsive gene). Of these, all showed greater than 1.5-fold increase in expression in at least one of the two replicates of the microarray hybridization (range of expression for *At103* and *BiP* were 1.3- to 1.7-fold and 1.3- to 2.1-fold, respectively). *Tubulin, GASA4,* and the *Histone* genes showed consistent differential expression in both experiments (2.6- to 2.9-fold, 1.7- to 2.2-fold, and 3.3- to 4.6-fold, respectively). These results indicate that heterologous microarray experiments may produce some false negatives

Table 1. Identification of genes induced in underground buds of leafy spurge upon dormancy release. Differential expression was confirmed by northern blot analysis for all of the genes listed (data not shown).

Differential display	Random sequencing of cDNA	Microarrays
Leucine-zipper protein	*Histone H3*	Adenosylhomocysteinase
Histone 2A	*Tubulin*	Guanine nucleotide binding protein
40S ribosomal protein	*GASA* (GA-responsive)	ATP synthase
Catalase	*BiP* (Glucose responsive)	Ubiquitin conjugating enzyme
Unknowns	*Lhcb*	Glyceraldehyde 3-phosphate dehydrogenase
		Unknown *Arabidopsis* 195F3T7

but few false positives if analyzed using > 1.5-fold increase in expression in both the biological replications as an indication of a positive result.

Timing of Expression of Selected Genes Following Dormancy Release

The timing of induction for several genes was characterized by northern blot analysis (Figure 1). Results from these experiments demonstrate that glucose- and GA-responsive genes and *Histone H3* show an increase in expression levels between 24 and 36 h after growth induction in all the sample sets tested. *Tubulin* expression does not consistently increase until approximately 12 h later (between 36 and 48 h after growth induction) and the light harvesting chlorophyll-binding protein gene (*Lhcb*) is induced 36 h later (72 h after growth induction). On the basis of the expression of *Histone H3* as an indicator of cell cycle state, these experiments show that cells within the underground buds initiate cell division and enter S-phase within 36 hr after defoliation and begin to show signs of photomorphogenesis within 72 h after growth induction. Also, GA signaling is initiated concomitantly with signals required for the induction of the S-phase of the cell cycle.

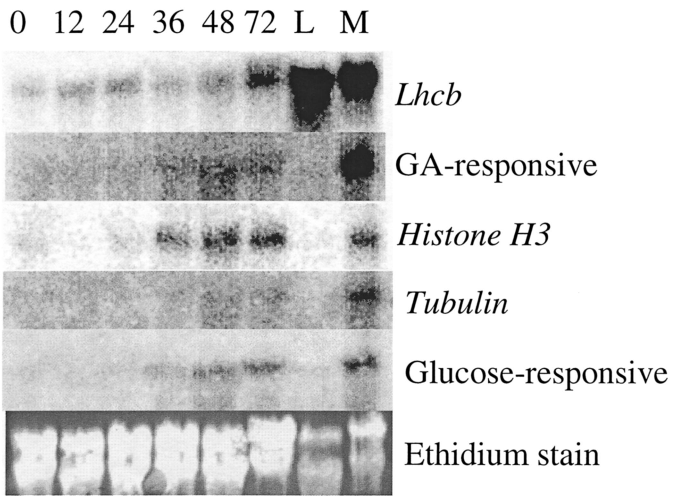

Figure 1. Northern blots of RNA from underground buds collected at various times (in hours) following defoliation-induced growth induction and from mature leaves (L) and growing meristems (M). Blots were sequentially probed with the selection of the designated clones and visualized using a Packard Instant Imager.

S-phase of Cell Division and GA Responses are Controlled by Leaf-derived but not Bud-derived Signals

Northern blot analysis was used to study the induction of selected genes following removal of specific plant organs known to be the source of signals controlling underground bud growth (Figure 2). Results from these experiments show that *Histone H3* and the GA- and glucose-responsive genes are upregulated after the removal of leaves but not after loss of either the apical meristem and axillary buds. However, loss of both leaves and apical and axillary buds are required for the induction of *Tubulin* and *Lhcb*. These data indicate that loss of the sugar signal produced by mature leaves may be sufficient for induction of GA responses and S-phase but not for the induction of later stages of cell division or photomorphogenesis.

GA and Auxin Signals Control Cell Division and Development Independently

Northern blot analysis was used to study the effects of exogenous GA and the auxin transport inhibitor (NPA) application on underground bud growth and development. Results from these experiments demonstrate that exogenous application of GA is sufficient for the induction of *Histone H3* and has some influence on the induction of *Lhcb* (Figure 3). However, both NPA treatment and exogenous application of GA is required to induce *Tubulin* and *Lhcb* expression to levels observed in growing underground buds 3 d after defoliation. The glucose-responsive gene appeared to be weakly induced by GA and NPA treatments. These findings suggest that GA is sufficient for the induction of the S-phase but as shown with the selective excision of plant organs, auxin is independently capable of inhibiting cell division and photomorphogenesis at a point in the cell cycle after the S-phase in underground buds of leafy spurge. It is difficult to explain the expression pattern observed with the glucose-responsive gene. Clearly it is not induced in underground buds following removal of the auxin producing organs but it does appear to be induced upon blocking of polar auxin transport. It is possible that additional signal(s) produced by selective excision of organs such as wounding may influence the expression of this gene.

Development of Novel Weed Control Methods Using Information on Signal Transduction Pathways

It should be possible to identify key *cis*-acting regulatory sequences from genes responding to signals that control un-

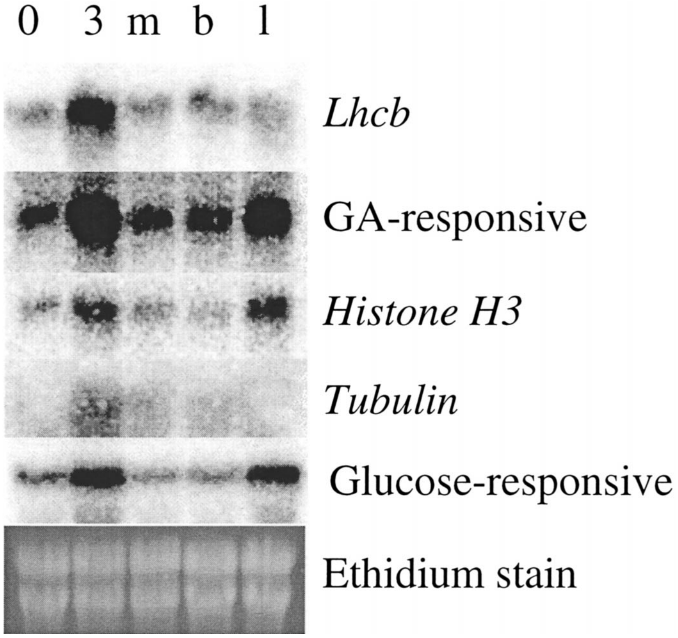

FIGURE 2. Northern blots of RNA from underground buds collected from control plants (0), 3 d following selective excision of the entire aerial portion of the plant (3), the apical meristem (m), the apical meristem and the axillary buds (b), and the apical meristem and the remaining leaves (l). Blots were sequentially probed with the designated clones and visualized using a Packard Instant Imager.

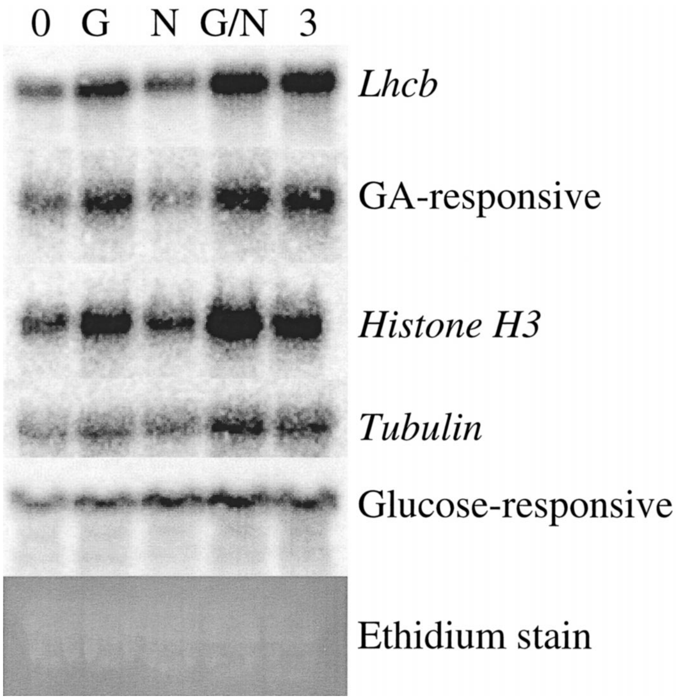

FIGURE 3. Northern blots of RNA from underground buds collected from control plants (0), 3 d following selective excision of the entire aerial portion of the plant (3), or after treatment with exogenous GA (G), NPA (N), GA and NPA (G/N). Blots were sequentially probed with the designated clones and visualized using a Packard Instant Imager.

derground bud growth. *cis*-Acting regulatory elements may be used to clone genes encoding additional components of regulatory pathways controlling underground bud dormancy and growth. Characterization and cloning of genes encoding the components of the signaling pathway should provide the information and tools needed to produce compounds or develop practices that will allow manipulation of these signals and result in novel methods to control leafy spurge.

There is an indication that crosstalk exists between signaling pathways controlling growth of underground buds and those involved in wounding, senescence (data not shown), and developmental state (Nissen and Foley 1987). These findings, if substantiated, could be used to predict synergistic actions of various herbicides and suggest optimal timing of herbicide application in conjunction with plant age or pre and post treatments, such as burning, mowing, or insect attack. For example, it is known that underground buds of older plants appear to be less responsive to the auxinic signals produced by the shoot apices. Thus, herbicide treatment following feeding by sheep or goats just prior to flowering should be more effective than herbicide treatments earlier in the season. Removal of the leaves alone by grazing should induce growth in underground buds under these conditions and make the plants more susceptible to herbicide damage. Also, the apparent reduction in auxin response might suggest that herbicides with an auxinic mode of action may be less effective later in the season than they are just prior to flowering.

Identification of regulatory proteins that interact with *cis*-acting elements in genes required for growth should prove to be useful targets for development of designer herbicides. With the increased knowledge and sophistication of enzyme structure and function, this approach is already considered mainstream by the pharmaceutical industry and is already being initiated at several commercial laboratories for identification of possible plant herbicides (Ascenzi et al. 2001). As chemical identification and production technology becomes cheaper and easier and demand for herbicides with limited environmental affect and greater species specificity increases, it may become increasingly economical to produce specific designer herbicides for specific weeds or environments.

Finally, with the possibility of using viral-induced gene silencing, it should be possible to block the expression of key proteins required to maintain dormancy in underground buds or seeds of leafy spurge and thus effectively inhibit a perennial growth cycle in this and possibly other weeds. Genes shown to be involved in regulation or required for growth of underground buds can be used to engineer viral-based biocontrol agents. Because of the nature of viral-induced gene silencing (requiring tight sequence specificity), it may be possible to design biocontrol agents that are very limited in the species or even ecotype that are effected, even if the viral host range is reasonably broad.

Conclusions

Previous work has demonstrated that two separate signals are produced, one by the mature leaves and another by growing shoot apices (apical or axillary meristems). Either is capable of inhibiting growth of underground buds in leafy spurge (Horvath 1999). We have used several techniques including differential display, analysis of randomly sequenced cDNA, and heterologous probing of Arabidopsis microarrays to identify a number of genes that are preferentially expressed in underground buds of leafy spurge as they break dormancy and resume growth. Further analysis of several such differentially expressed genes has provided evidence that the leaf-derived signal acts through the induction of GA signaling to specifically inhibit cell division at the G_1/S-phase transition, either by inhibiting GA synthesis or inhibiting GA signal transduction in the underground buds. The meristem-derived signal is likely auxin and controls growth of underground buds at a later stage in cell division. Both signals must be blocked or overcome to induce full growth and development of underground buds. Future work should include cloning and characterization of genomic copies of the differentially expressed genes and characterization of their regulatory sequences. This information should enhance our ability to predict the effectiveness of various weed control strategies and allow the development of novel weed control techniques.

Sources of Materials

[1] Commercial potting soil, Sunshine Mix, Sun Gro Horticulture Inc., Bellevue, WA 98008.

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